Effects of Sulfite on the Uptake and Binding of Benzo[*a*]pyrene Diol Epoxide in Cultured Murine Respiratory Epithelial Cells

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Sulfur dioxide (SO_2) may act as a cocarcinogen with benzo[*a*]pyrene (BaP) in the respiratory tract. We have modeled this effect by examining the interactions of 7r,8t-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE) with sulfite, the physiological form of SO_2, in a murine respiratory epithelial cell line (C10). We exposed C10 cells to [^{3}H]-*anti*-BPDE and determined the effects of 1 and 10 mM sulfite on the uptake and subcellular localization of labeled products. Autoradiographic analysis showed that sulfite doubled the nuclear localization of *anti*-BPDE-derived materials after a 4-hr incubation period. The net nuclear localization of *anti*-BPDE-derived materials was not affected by sulfite during the first 60 min, but nuclear localization continued to increase in the sulfite-containing incubations throughout the 4-hr incubation period. Little increase in nuclear localization of *anti*-BPDE-derived material was noted in the incubations without sulfite after 60 min. Subcellular fractionation was performed to determine the amount of label associated with cytosolic and nuclear fractions and to determine covalent binding to protein and DNA. Sulfite produced a modest increase in the amount of [^{3}H]-*anti*-BPDE-derived products bound to protein; however, binding to nuclear DNA increased by more than 200% with 10 mM sulfite. Analysis of the supernatants from the cytosolic and nuclear fractions of cells exposed to *anti*-BPDE and sulfite demonstrated the presence of 7r,8t,9t-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-10c-sulfonate (BPT-10-sulfonate). [^{3}H]-BPT-10-sulfonate was unable to enter C10 cells, suggesting that it is formed intracellularly. Once formed, this compound may be unable to leave the cell. The observed intracellular formation of BPT-10-sulfonate, a more stable DNA-modifying BaP derivative than BPDE and one which probably cannot leave the cell, could be responsible for this extended time course of nuclear localization and DNA modification. *Key words*: benzo[*a*]pyrene, benzo[*a*]pyrene diol epoxide, DNA modification, sulfite, type II cells. *Environ Health Perspect* 102:216–220(1994)

Benzo[*a*]pyrene (BaP) and sulfur dioxide (SO_2) are common industrial and environmental contaminants. Epidemiological evidence suggests that high levels of SO_2 increase the incidence of respiratory tract carcinoma in humans (*1,2*). Further, chronic concurrent inhalation exposure to SO_2 and BaP results in an increased incidence of upper respiratory tract neoplasia in rats (*3*) and hamsters (*4*) over that seen with BaP alone. These findings suggest that SO_2 is a cocarcinogen for BaP in the respiratory tract. We have investigated the chemical, metabolic, and genotoxic interactions of SO_2, in the form of the hydrated sulfite anion, with the BaP metabolites (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BaP-7,8-diol)(5-7) and (±)-7r,8t-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE)(*6,8–10*). In subcellular systems, sulfite-derived reactants can both oxygenate BaP-7,8-diol to convert it to *anti*-BPDE (5) and also add to BP-7,8-diol to produce a sulfonate derivative (*7,11*). Sulfite, a potent nucleophile, also efficiently attacks *anti*-BPDE to produce 7r,8t,9t-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene-10c-sulfonate (BPT-10-sulfonate)(*8*). Although it is far more stable than *anti*-BPDE, BPT-10-sulfonate retains the ability to bind covalently to DNA (*8*). Sulfite thus not only can increase the formation of the highly reactive alkylating agent *anti*-BPDE, but it can also produce an additional class of alkylating agents, the BaP triol sulfonates (*7,8*).

These reactions between sulfite and BaP derivatives also occur in the presence of viable cells. In bacterial mutagenicity assays using *Salmonella typhimurium* strains TA98 and TA100, sulfite activates BaP-7,8-diol as a mutagen (*6,7*), and strongly potentiates the mutagenicity of *anti*-BPDE (*6,8–10*). The ability of sulfite to enhance the genotoxicity of mutagenic BaP derivatives provides a plausible mechanism for the observed cocarcinogenic effect of SO_2 with BaP (*6–9*). A critical question, however, is whether these same reactions can occur in the more complex milieu of an intact mammalian cell system. We have addressed this question by examining the interactions of sulfite and *anti*-BPDE in cultured murine respiratory epithelial cells. The Balb/c-derived C10 cell line was chosen as an appropriate system for these studies. This is a stable respiratory epithelial cell population with characteristics similar to those of alveolar type II cells (*12*). We have used this system to examine both the intracellular and extracellular chemical interactions of *anti*-BPDE and sulfite and to examine the effects of these interactions on the uptake and nuclear localization of, and on the covalent modification of cellular macromolecules by, *anti*-BPDE-derived species. Our findings demonstrate the formation of BPT-10-sulfonate in a viable mammalian cell system and suggest the possible significance of this formation in regard to the modification of nuclear DNA and possible mutagenic consequences.

Materials and Methods

Chemicals

Racemic *anti*-BPDE and randomly labeled [^{3}H]-*anti*-BPDE (specific activity, 1430 mCi/mmol) were supplied by the National Cancer Institute repository and dissolved in anhydrous silylation grade tetrahydrofuran (THF). THF and dimethylsulfoxide (DMSO) were purchased from Pierce Chemical Co. (Rockford, Illinois); 99+% triethylamine (TEA) was from Aldrich Chemical Company (Milwaukee, Wisconsin). All other chemicals and solvents were purchased from Fisher Scientific (St. Louis, Missouri) unless otherwise specified. Hydrolysis of *anti*-BPDE to obtain the isomeric 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrenes (BaP tetraols) standards was carried out as described previously (*13*). Synthesis and characterization of BPT-10-sulfonate and [^{3}H]-BPT-10-sulfonate (specific activity, 57 mCi/mmol) was accomplished as previously described (*8*). We carried out all procedures under subdued light. Synthetic products were stored as dry solids under nitrogen at -20°C. Stock solutions used in incubations were prepared fresh daily in dry DMSO. All stock solutions were determined to be >99% pure by HPLC. Analytical HPLC was conducted using a 4.6 × 250 mm Ultrasphere ODS column (Beckman Instruments Co., Fullerton, California) eluted with a methanol-water gradient (*8*). Detection consisted of monitoring absorbance (344 nm) and fluorescence (344 nm excitation, 380 nm emission).

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Cell Cultures

C10 cells, derived from type II respiratory epithelial cells from Balb/c mice, were provided by Gary Smith (University of New South Wales) (*12*). The cells were grown in CMRL 1066 (Gibco Inc., Bethesda, MD) medium containing 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% fetal bovine serum, at 37°C in an atmosphere of 5 % CO_2.

Uptake and Localization of Labeled BaP Derivatives

We seeded eight-well cell culture slides (Nunc Inc., Naperville, Illinois) with ≈ 4×10^3 cells/well and incubated them as described above for 24 hr to achieve 80-90 % confluent monolayers. The medium was replaced by sterile phosphate-buffered saline (PBS) and [^{3}H]-*anti*-BPDE, diluted with unlabeled diol epoxide to a specific activity of 50 µCi/µmol, or [^{3}H]-BPT-10-sulfonate (57 µCi/µmol) solutions in DMSO were added. DMSO concentration was held constant at 1 % (v/v). Where indicated, we added sterile solutions of sulfite in PBS 3 min before the addition of [^{3}H]-*anti*-BPDE. Total volume for all wells was 250 µl. All slides were incubated at 37°C under 5% CO_2. After the incubation period, we removed the PBS for analysis by HPLC and washed the wells gently three times each with 250 µl of 50% aqueous methanol to remove unbound BaP derivatives. The cells were fixed for 1 hr in 4% aqueous glutaraldehyde, washed with distilled water, and air dried. The slides were coated with Kodak NTB-2 liquid photographic emulsion and exposed for approximately 28 days at 0°C. After development with Kodak Dectol developer, the cells were stained with a hematoxylin:eosin stain. Resolution of cells and automated grain counts were accomplished under darkfield illumination using a Nikon Type 108 Analytical Microscope. Automated data collection was accomplished using the Image Analysis System (Analytical Imaging Concepts, Atlanta, Georgia). We quantitated the extent of nuclear localization by microscopic examination (at 1000×) and counting developed silver grains over the nuclei of at least 20 randomly selected cells. Background grain counts were made in areas directly adjacent to the cells, and the values were used to correct the counts for the cells. Results are expressed as the means ± SD for counts from a single well.

Subcellular Localization of [^{3}H]-*anti*-BPDE-Derived Products

C10 cells were grown to confluent monolayers in CMRL 1066 medium as described (approximately 3×10^6 cells/100 mm plate). The cells were gently washed with PBS, and an aliquot of sterile PBS was layered over the cells. We then added a DMSO solution of [^{3}H]-*anti*-BPDE (50 µCi/µmol) then for a final concentration of 1 µM [^{3}H]-*anti*-BPDE. The DMSO content of the incubations was always 1% (v/v). Where indicated, aliquots of aqueous sodium sulfite were added 3 min before addition of [^{3}H]-*anti*-BPDE to give a final sulfite concentration of 1 or 10 mM. The final volume of PBS and additions was 3.0 ml. The cells were then incubated at 37°C in an atmosphere of 5% CO_2/95 % room air. At the conclusion of each incubation, we removed PBS and analyzed for metabolite content by HPLC. Cells from three replicate plates were washed with PBS, harvested, pooled, and resuspended in 50 ml of PBS.

We isolated nuclei from this cell suspension following the method of Schildkraut and Maio (*14*). Briefly, the cells were pelleted by centrifugation at 1500*g* for 20 min, resuspended in 25 ml of 0.01 M Tris, pH 7.4 containing 0.3 mM $CaCl_2$ and 0.5% Triton X-100, and incubated at 0°C for 10 min. The cells were then disrupted using a Potter-Elvejhem homogenizer. Light microscopy of this homogenate revealed that greater than 85% of the cells were lysed. We centrifuged the homogenates at 300*g* for 10 min, and the resulting cytosolic fraction was removed for analysis of total radioactivity by liquid scintillation counting, protein content (*15*), and covalent binding (*16*), and *anti*-BPDE metabolite content (*8*). The crude nuclear pellet was resuspended in 8.75 ml of 2.0 M sucrose containing 1.5 mM $CaCl_2$ and buffered with 0.01 M Tris, pH 7.4, and then layered onto a 5.25 ml cushion of 2.2 M sucrose in an ultracentrifuge tube. This sample was centrifuged at 50,000 *g* for 1 hr. The supernatant was discarded and the pellet resuspended in 1.0 ml PBS. We assessed purity of the nuclear preparation using phase-contrast microscopy. Aliquots (100 µl) of the nuclear fraction were assessed for radioactivity, metabolite content, and protein binding as described above. The remainder of the nuclear fraction was retained for DNA isolation. DNA was isolated by phenol-chloroform extraction, RNAse A treatment, and ethanol precipitation. The purified DNA was lyophilized and redissolved in 10 mM NaCl-1 mM EDTA, quantitated by absorbance at 260 nm (20 AU/mg DNA/ml), and the bound labeled material was then quantitated by liquid scintillation counting. All incubations were run in triplicate, and results are reported as means ± SDs.

Results and Discussion

Effects of Sulfite on the Cellular Localization of [^{3}H]-*anti*-BPDE

The effect of sulfite on the cytoplasmic and nuclear localization of [^{3}H]-*anti*-BPDE in C10 cells was first examined by autoradiographic methods. Results were quantified by automated image analysis and data collection of exposed silver grains associated with nuclei. Initial studies examined the concentration dependence for this nuclear localization and its enhancement by sulfite. In cells exposed for 4 hr to [^{3}H]-*anti*-BPDE alone, a concentration-dependent increase in nuclear localization was noted over the concentration range of 0.05–1.0 µM (Fig. 1). Upon inclusion of 1 or 10 mM sulfite, there was an increase of up to 86% in the level of nuclear localization of [^{3}H]-*anti*-BPDE-derived material at all concentrations of [^{3}H]-*anti*-BPDE over that seen in the absence of sulfite (Fig. 1). The extent of enhancement of *anti*-BPDE localization elicited by 1 and 10 mM sulfite was identical. The diol epoxide is readily able to partition across membranes to reach all cellular compartments. The increased nuclear localization relative to other organelles may well result from the noted specificity for covalent binding to double-stranded DNA exhibited by *anti*-BPDE (*16*). Clearly, a substantial increase in nucleus-associated label resulted from this treatment with sulfite. We have reported previously that sulfite increased the covalent modification of DNA by *anti*-BPDE and the resultant genotoxicity in bacterial systems (*9*), and the data shown here support a similar interaction in a mammalian cell system.

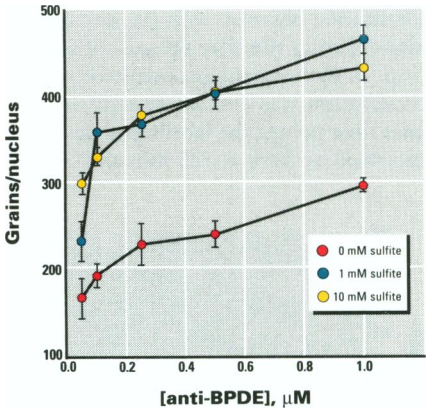

Figure 1. Effect of sulfite on the nuclear localization of [^{3}H]-*anti*-BPDE-derived products. C10 cells were incubated for 4 hr at 37°C with [^{3}H]-*anti*-BPDE and sulfite. The cells were fixed and dried, and autoradiograms were prepared as described in Materials and Methods. Following development, the cells were stained with hematoxylin-eosin, and the extent of nuclear localization was visualized by microscopic examination. The extent of nuclear localization was quantitated by microscopic examination (at 1000×) and counting developed silver grains over the nuclei of at least 20 randomly selected cells. Background grain counts were made in areas directly adjacent to the cells, and the values were used to correct the counts for the cells. Data are expressed as the means ± SDs of all corrected nuclear counts.

Kinetics of Sulfite-mediated Nuclear Localization of [^{3}H]-*anti*-BPDE

We examined the kinetics of *anti*-BPDE uptake and the effects of sulfite on this process using 0.1 μM for the diol epoxide and 1 mM for sulfite. The concentration of diol epoxide was chosen based both on the potent stimulation of nuclear localization due to sulfite at that concentration (Fig. 1) and on the position of this diol epoxide concentration within the reported dose–response range for mutagenicity in mammalian cell systems (*17–19*). We chose the sulfite concentration based on the equal effects of 1 and 10 mM sulfite observed at the 4-hr time point (Fig. 1) and on our previous experience with sulfite and *anti*-BPDE in bacterial test systems (*6,8–10*). The half-life of *anti*-BPDE in aqueous systems is about 2 min (*18*), indicating that uptake, distribution, and covalent binding of the diol epoxide must take place rapidly. The products of these reactions will then be either retained within the cell or eliminated, depending on the relative polarity of the products. In control incubations exposed only to *anti*-BPDE, there was an initial burst of nuclear localization during the first 15 min of incubation, with a more gradual increase between 15 and 60 min (Fig. 2). Between 60 and 240 min, only a 44% increase in nuclear labeling was observed. The net nuclear localization of diol epoxide-derived materials in the presence of 1 mM sulfite was indistinguishable from the extent of net localization observed with the diol epoxide alone during the first 60 min of incubation. A distinct enhancement of nuclear localization was elicited by sulfite at the later time points: the labeling of nuclei was increased by 31% at 120 min and by 88% at 240 min relative to the levels observed in the absence of sulfite. Although these data do not rule out effects of sulfite on cellular components leading to this increased nuclear localization, our findings also are consistent with the formation of a more stable species than *anti*-BPDE, which possesses reactivity or structural features that favor its accumulation in the nucleus and inhibit its release into the extracellular environment. Such properties are in agreement with the reported reactivity and apparent polarity of BPT-10-sulfonate (*8,10*).

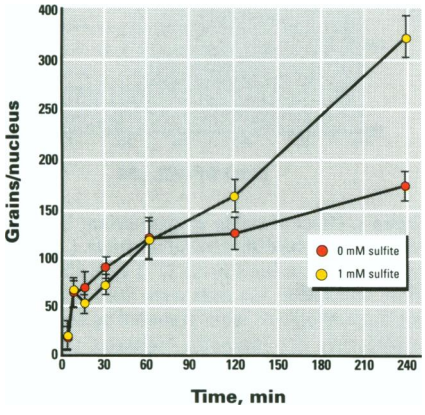

Figure 2. Sulfite and the kinetics of nuclear localization of [^{3}H]-*anti*-BPDE-derived products. C10 cells were exposed to 0.1 μM [^{3}H]-*anti*-BPDE (50 μCi/μmol) in the presence of 0 or 1 mM sulfite. Incubations were stopped after 3, 7, 15, 30, 60, 120, and 240 min. The cells were then washed, fixed, and analyzed by autoradiography as described in Materials and Methods and in Figure 1.

Effects of Sulfite on the Subcellular Distribution and Macromolecular Binding of *anti*-BPDE

In regard to genotoxicity, the effects of sulfite on the behavior of *anti*-BPDE within the nucleus and resultant covalent binding to DNA are of great interest. C10 cells from incubations similar to those described above were lysed and fractionated by centrifugation to produce a cytosolic and a nuclear fraction. Covalent binding of labeled materials to protein from each of these fractions was determined, and covalent modification of DNA from the nuclear fraction also was measured. C10 cells exposed to 1 μM [^{3}H]-*anti*-BPDE and either 1 or 10 mM sulfite showed modest increases in the levels of cytosolic and nuclear protein binding of [^{3}H]-*anti*-BPDE-derived products over that seen in the absence of sulfite (Table 1). Analysis of the protein binding data by one-way ANOVA followed by Fisher's least significant difference analysis established that the increase elicited by 10 mM sulfite in both cytosolic and nuclear protein binding was statistically significant ($p<0.05$), but not at 1 mM sulfite. The effects of sulfite on DNA binding, however, were far more impressive. When 1 mM sulfite was included, resultant DNA modification was increased by 130%, and 10 mM sulfite elicited a 210% increase in the level of DNA binding over that observed with [^{3}H]-*anti*-BPDE alone (Table 1). This increased binding of [^{3}H]-*anti*-BPDE-derived products to both the nuclear protein fraction and DNA demonstrates effects of sulfite consistent with an enhanced genotoxic response.

We analyzed the supernatants from the cytosolic and nuclear fractions by HPLC to characterize the stable end products derived from [^{3}H]-*anti*-BPDE. Representative radiochromatograms are shown in Figure 3. In the absence of sulfite, both the cytosolic and the nuclear fractions contained the two diastereomeric BaP tetraols formed by the spontaneous hydrolysis of *anti*-BPDE. No detectable glutathione conjugates of the diol epoxide were seen (t_R = 14.7 min). Sulfite reduces the scavenging efficiency of the cellular glutathione system by depleting reduced glutathione (*9,20*) and by inhibiting the enzyme glutathione-*S*-transferase (*20,21*). Such inhibition of glutathione-dependent pathways has been advanced as a possible explanation for the increased mutagenicity and cytotoxicity of BaP derivatives in cell systems (*20–24*). It is possible that sulfite inhibits the glutathione-*S*-transferase system in C10 cells, thus increasing the effective concentration of *anti*-BPDE. The lack of detectable glutathione–BPDE conjugates in the subcellular fractions from these cells or in the extracellular medium (data not shown) argues against a critical role for this conjugation pathway. This apparent diminished role for glutathione-dependent detoxication of *anti*-BPDE in C10 cells does not agree with reports from some other mammalian cell systems (*20,22–24*), but it does agree with our observations in bacterial systems (*8,9*). Moreover, mammalian cell types are known in which glutathione-dependent pathways are not important modulators of diol epoxide toxicity (*25*).

Analysis of subcellular fractions from cells treated with either 1 or 10 mM sulfite demonstrated not only the formation of BaP tetraols, but also the presence of BPT-10-sulfonate (t_R = 10,11 min). The presence of the BaP tetraols in both cytoplasmic and nuclear fractions indicates that the relatively lipophilic *anti*-BPDE readily partitions into cells and into the nucleus. The presence of BPT-10-sulfonate in fractions derived from cells exposed to *anti*-BPDE and sulfite indicates either that the formation of BPT-10-sulfonate occurs within the cell or that BPT-10-sulfonate formed in the extracellular environment is able to enter intact cells.

Table 1. Protein and DNA binding of *anti*-BPDE-derived materials

Fraction	Sulfite, mM	Protein binding, pmol/mg	DNA binding, pmol/mg
Cytosolic	0	80 ± 6	ND
	1	93 ± 10	ND
	10	108 ± 7	ND
Nuclear	0	76 ± 6	4.8 ± 1.5
	1	86 ± 10	11.1 ± 0.3
	10	111 ± 12	14.9 ± 0.6

ND, not determined. Fractions were isolated and protein and DNA binding measured as described in Materials and Methods. Data represent the means ± SDs of triplicate determinations.

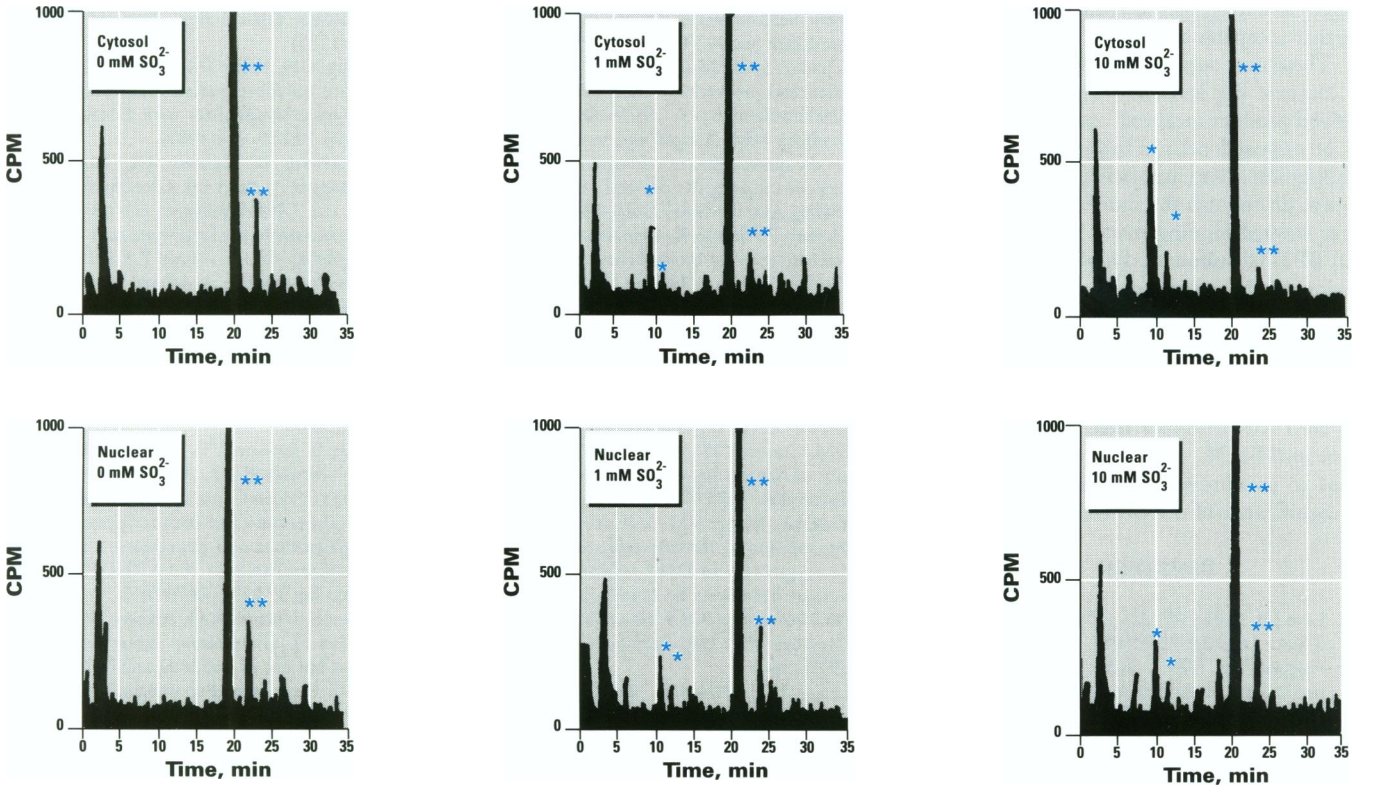

Figure 3. HPLC analysis of [^{3}H]-*anti*-BPDE-derived products. Supernatants from the cytosolic and nuclear fractions from C10 cells exposed to 1 μM [^{3}H]-*anti*-BPDE in the presence of 0, 1, and 10 mM sulfite were analyzed by HPLC, as described in Materials and Methods. Product identity, based on retention time: t_R = 3 min, impurity in [^{3}H]-*anti*-BPDE; (*) 10, 11 min = BPT-10-sulfonates; (**) 20, 23 min = BaP tetraols. The tetraols eluting at 20 and 23 min are known to be the *trans*- and *cis*- diastereomers, respectively. The same relationship is assumed for the two BPT-10-sulfonates, which elute at 10 and 11 min (*8*).

Uptake and Nuclear Localization of [^{3}H]-BPT-10-sulfonate

The site of formation of BPT-10-sulfonate and the ability of this BaP derivative to cross cellular membranes are critical issues to address in regard to the mechanisms and significance of sulfite–diol epoxide interactions. We examined the ability of BPT-10-sulfonate to cross cellular membranes by exposing C10 cells to [^{3}H]-BPT-10-sulfonate over an extracellular concentration range of 1–20 μM. Autoradiograms, prepared and analyzed as described above, demonstrated that for concentrations of 1–10 μM BPT-10-sulfonate, there was no significant nuclear localization of labeled materials (Fig. 4). At a concentration of 20 μM BPT-10-sulfonate, a fourfold increase above background was noted in the number of grains/nucleus. This apparent nuclear localization is less than 7% of the nuclear localization observed with *anti*-BPDE alone (Fig. 1), and about 4% of the level obtained with *anti*-BPDE in the presence of sulfite (Fig. 1), despite the fact that the BPT-10-sulfonate concentration was 80-fold higher than the *anti*-BPDE concentration chosen for this comparison. This result is consistent with the inability of BPT-10-sulfonate to partition into octanol from an aqueous milieu (Green JL, unpublished observations), as well as our previously reported findings regarding the bacterial mutagenicity of these compounds (*7–9*). BPT-10-sulfonate simply cannot enter intact cells, whereas *anti*-BPDE and sulfite do enter cells efficiently. This inability of BPT-10-sulfonate to cross membranes supports two critical corollaries relating to the disposition of BaP derivatives in the presence of sulfite. The first is that any BPT-10-sulfonate found in intracellular fractions, such as the cytosolic and nuclear fractions examined here, must result from the intracellular reaction between sulfite and *anti*-BPDE. Second, if BPT-10-sulfonate is formed within a cell, it very likely cannot be excreted from that cell.

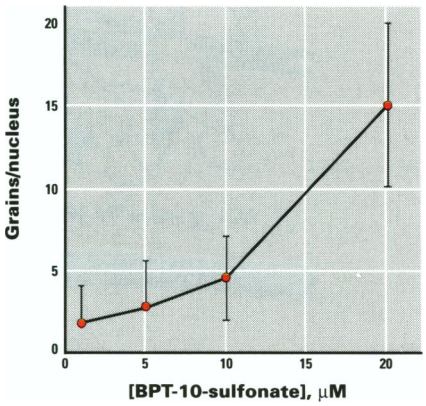

Figure 4. Nuclear localization of [^{3}H]-BPT-10-sulfonate. C10 cells were treated with 1 to 20 μM [^{3}H]-BPT-10-sulfonate for 4 hr and analyzed as described in Figures 1 and 2. Data are expressed as the means ± SDs.

In summary, C10 murine respiratory epithelial cells exposed to *anti*-BPDE and sulfite show enhanced nuclear localization of *anti*-BPDE-derived material. Results from examination of the time course of net nuclear localization of diol epoxide-derived species indicate that sulfite does not alter this process detectably during the first hour, but rather that it extends the linear increase in net localization between 1 and 4 hr. The presence of sulfite increased the covalent modification of DNA by diol epoxide-derived species up to threefold. Detection of BPT-10-sulfonate in cytosolic and nuclear fractions derived from cells exposed to both *anti*-BPDE and sulfite demonstrates the presence of another potentially reactive species in addition to the highly reactive epoxide. The inability of [^{3}H]-BPT-10-sulfonate to partition across membranes may indicate that it is unable to be eliminated from the cell, thereby increasing the exposure to a more stable reactive intermediate than *anti*-BPDE. The net effect of this sequestration is the enhanced exposure of critical cellular

targets to a longer-lived reactive intermediate that is capable of binding to DNA.

These data support the ability of sulfite to increase the level of [^{3}H]-*anti*-BPDE-derived product localized within the nucleus of exposed cells. Unfortunately, the binding levels and autoradiographic studies do not distinguish the individual contributions toward binding made by *anti*-BPDE and BPT-10-sulfonate. Detailed characterization of the structures of DNA adducts formed by BPT-10-sulfonate and on the sequence specificity for their formation are underway. If specific marker adducts or a pronounced sequence specificity is found for BPT-10-sulfonate that distinguish it from *anti*-BPDE, then the tools will be in hand to conclusively assess the toxicological significance of this novel derivative.

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